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TITLE: Pilot Comparison of Stromal Gene Expression among Normal Prostate Tissues and Primary Prostate Cancer Tissues in White and Black Men

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14. ABSTRACT
This hypothesis development project tested the feasibility of identification, laser capture microdissection, and expression analysis of prostate-stroma specific cells in normal and cancerous prostates, and aims to develop preliminary data sufficient to identify potential differences in stromal RNA expression in normal and cancerous prostate tissue. Our studies found that it is difficult but not impossible to histologically identify prostate zones with an acceptable degree of confidence in frozen tissues, eliminating the need to attempt expression studies in fixed tissues with their attendant biases. LCM of stromal tissue was completed for 6 normal prostates from men across the age range and of the two racial groups studied, and from prostates from men of similar ages with adenocarcinoma identified distant from the area of dissection. High Quality RNA was isolated, and duplicate Affymetrix Plus 2.0 chip analysis performed. We observe potentially significant expression differences in both normal epithelial and stromal cells from fully normal prostates as compared to prostates containing adenocarcinoma which are now subject to formal hypothesis testing.

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Introduction

Recent advances in prostate biology suggest that stromal cells surrounding prostate epithelia may play a key role in permitting or stimulating epithelial cells to lose control and form precancerous and cancerous lesions. The goal and purpose of this Hypothesis Development project is to obtain preliminary data sufficient to begin to explore the role of prostate stromal cells in prostate carcinogenesis under conditions as rigorously controlled as current technology allows.

Body

Please note that because this is a Hypothesis Development Award project, no statement of work was required or provided as part of the original funded proposal. Nonetheless, the primary goal and steps required to complete the project were clearly identified in the original application.

As in any well-managed research project, we have kept our eyes on the goal, while changing tactics and methods to adjust to our own and outside research progress as the study progressed.

The following is a list of the main steps taken toward reaching the goal of this study:

1. Histologic analysis of available normal prostate sections from frozen and fixed (alcohol or formalin) tissue to identify tissue blocks which we can reliably be identified as containing prostate anatomic subzones (Central Zone, Peripheral Zone, Transition Zone, as illustrated in Figure 1). This was covered in our interim study reports. This was far more time-consuming than expected, but was completed successfully together with our prostate histology expert Dr. De Marzo.

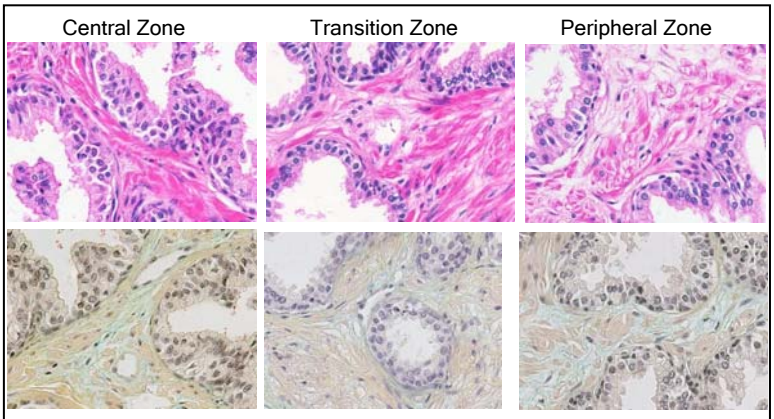

Figure 1: Representative histology from 13 normal prostates collected. H&E (upper row) and Movat's (lower row) stained sections from PZ, TZ, and CZ. All images are from alcohol-fixed, paraffin-embedded sections. Movat's stains smooth muscle pink-red, and ground substance blue-green.

2. Decision to use only frozen tissues for RNA expression analysis for this pilot study based on discussion and careful review of current literature on using fixed tissues for RNA analysis. While many have reported the use of fixed tissues for RNA analysis, this method has limitations which should be avoided if sufficient frozen tissues are available. Analysis of the number and type of available tissues showed that we likely have sufficient frozen normal prostate for hypothesis development. If successful, this eliminates one layer of potential data confounding.

3. Continued work on improving function of our integrated database application to manage data emanating from the project. Our main focus has been on integrating RNA expression results with Tissue Microarray staining results. Because most current studies linking phenotype (cancer/normal for example) with molecular data (expression of specific gene products for example) do not have database control of all of their data, most expression studies to date report large amounts of data but do not stand up to recurrent hypothesis testing. With every new study performed in our laboratory, we attempt to improve these linkages to allow our science to continue to improve. This was covered in our previous progress report.

4. Decision to complete the study in collaboration with the Laboratory of Cancer Genetics, National Cancer Institute, headed by Dr. Michael Emmert-Buck. Our laboratories have a long history of collaboration, and our research interests are complementary. Together, we decided to widen the scope of the study to compare both epithelial and stromal RNA expression.

   Six of the normal cases identified above and six cases containing tumor foci elsewhere in the gland (from the Emmert-Buck laboratory) were laser-capture microdissected for normal-appearing epithelium, and normal appearing stroma. RNA was then extracted using the PicoPure RNA Extraction Kit (Molecular Devices) and subjected to a quality and quantity control assessment. Quality of RNA was evaluated using an Agilent 2100 Bioanalyzer for RIN and 28S/28S ratios. Quantity was measured using a NanoDrop Spectrophotometer. Two rounds of linear oligo-dT amplification using the MessageAmp II Kit (Ambion) to amplify messenger RNA for GeneChip analysis. After a second Bioanalyzer step to confirm amplification, mRNA were fragmented and hybridized to oligo-nucleotide arrays (Affymetrix U133 Plus 2.0 GeneChips). The GeneChips contain over 54,000 probe sets and were run in technical replicates for quality assurance.

   Laser-capture microdissection went smoothly, with an average of 4,180 shots being taken for epithelial samples and 20,359 shots taken for stromal samples. A five-fold increase in shot number was necessary for stromal areas to increase RNA yield because it is a relatively cellular-poor region. Extracted RNA from each region averaged 13.49 ng/ul for the epithelium, and 8.33 ng/ul for the stromal samples. Quality assessment revealed 28S/18S ratios of 1.48 and 1.10 for the epithelium and stroma, respectively, and RINs (RNA Integrity Numbers) of 7.90 and 7.18, respectively as well, indicating high quality RNA suitable for expression array analysis. All of the gene chip hybridizations were completed as of Friday, January 19, 2007.

5. Analysis of Affymetrix expression data. The NCI mAdb array data analysis package was used to compare expression levels between groups. The most significant observations to emerge from these comparisons are summarized in the four tables below.

Table 1: Transcripts greater than 10-fold upregulated in normal epithelium from older subjects with prostate cancer when compared to normal epithelium from prostates from younger subjects without cancer.

Fold Change	Stand. Deviation	Gene	Affy Feature ID	Entrez GeneID	UniGene	hgB36_Probe Cytoband	Description
26.34	0.37	HES1	203394_s_at	3280	Hs.250666	3q29	hairy and enhancer of split 1, (Drosophila) (HES1), mRNA.
24.63	0.57	SOCS3	227697_at	9021	Hs.527973	17q25.3	suppressor of cytokine signaling 3 (SOCS3), mRNA.
17.95	0.29	VCAM1	203868_s_at	7412	Hs.109225	1p21.2	vascular cell adhesion molecule 1 (VCAM1), transcript variant 2, mRNA.
12.2	0.32	HIST1H4C	205967_at	8364	Hs.46423	6p22.1	histone cluster 1, H4c (HIST1H4C), mRNA.

12.08	0.36	CLU	222043_at	1191	Hs.436657	8p21.1	clusterin (CLU), transcript variant 1, mRNA.
11.2	0.31	SLC8A1	235518_at	6546	Hs.468274	2p22.1	solute carrier family 8 (sodium/calcium exchanger), member 1 (SLC8A1), mRNA.
10.69	0.35	LUM	201744_s_at	4060	Hs.406475	12q21.33	lumican (LUM), mRNA.
10.45	0.42	NR4A2	216248_s_at	4929	Hs.563344	2q24.1	nuclear receptor subfamily 4, group A, member 2 (NR4A2), transcript variant 4, mRNA.

Table 2: Transcripts greater than 5-fold upregulated in normal stroma from older subjects with prostate cancer when compared to normal stroma from prostates from younger subjects without cancer.

Fold Change	Stand. Deviation	Gene	Feature ID	hgB36_Probe Cytoband	UniGene	RefSeq	Entrez GeneID	Description
12.57	0.61	SOCS3	227697_at	17q25.3	Hs.527973	NM_003955	9021	suppressor of cytokine signaling 3 (SOCS3), mRNA.
10.09	0.51	ATF3	202672_s_at	1q32.3	Hs.460		467	Activating transcription factor 3
9.05	0.26	ADAM28	208268_at	8p21.2	Hs.174030	NM_021777	10863	ADAM metallopeptidase domain 28 (ADAM28), transcript variant 3, mRNA.
7.98	0.49		235739_at	2q24.1	Hs.656946			Transcribed locus
7.25	0.31	EGR3	206115_at	8p21.3	Hs.534313	NM_004430	1960	early growth response 3 (EGR3), mRNA.
7.16	0.45	ARHGAP15	244061_at	2q22.2	Hs.171011			Rho GTPase activating protein 15
6.91	0.39		1557459_at	11q23.1	Hs.673033			MRNA; cDNA DKFZp547O0210 (from clone DKFZp547O0210)
6.34	0.37	NR4A2	204622_x_at	2q24.1	Hs.563344	NM_173173	4929	nuclear receptor subfamily 4, group A, member 2 (NR4A2), transcript variant 4, mRNA.
6.01	0.17		238673_at	8q24.12	Hs.359393			Transcribed locus
5.61	0.33	LTB	207339_s_at	6p21.33	Hs.376208	NM_009588	4050	lymphotoxin beta (TNF superfamily, member 3) (LTB), transcript variant 2, mRNA.
5.28	0.37	C16orf54	1559584_a_at	16p11.2	Hs.331095	NM_175900	283897	chromosome 16 open reading frame 54 (C16orf54), mRNA.

Table 3: BioCarta and KEGG molecular pathway-based differences detected between normal epithelium from older subjects with prostate cancer and normal epithelium from prostates from younger subjects without cancer (red: upgregulated, green:downregulated)

# of Features	BioCarta Pathway
5	Keratinocyte Differentiation: EGF, EGFR, JUN, SP1, ETS1
4	Mechanism of Gene Regulation by Peroxisome Proliferators via PPARa(alpha): JUN, NR2F1, SP1, ACOX1

4	Agrin in Postsynaptic Differentiation: JUN, SP1, EGFR, ARHGEF6
4	MAPKinase Signaling Pathway: JUN, MEF2A, SP1, RAPGEF2
3	Signaling Pathway from G-Protein Families: JUN, GNAS, ASAH1
3	EGF Signaling Pathway: EGF, EGFR, JUN
3	METS affect on Macrophage Differentiation: JUN, ETS1, RBL1

# of Features	**KEGG Pathway**
14	Focal adhesion: CCND2, EGF, EGFR, JUN, CAV1, PARVA, COL1A1,COL1A2, COL3A1, COL5A1, COL5A2, COL6A3, LAMB1, TNC
11	MAPK signaling pathway: TGFB2, EGF, EGFR, JUN, RASGRF2, RAPGEF2, DUSP6, ZAK, FGFR2, ATF4, PPM1A
10	Cell Communication: LAMB1, TNC, DSC3, COL1A1, COL1A2, COL3A1, COL5A1, COL5A2, COL6A3, KRT18
10	Cell adhesion molecules (CAMs): MCAM, NCAM1, VCAN, VCAM1, CLDN11, CNTN1, PTPRC, HLA-DPB1, HLA-DPA1, ALCAM
9	Leukocyte activation: BCL11A, CXCR4, PAWR, PTPRC, LCP2, KLF6, CX3CL1, EGR1, TPD52
9	ECM-receptor interaction: LAMB1, TNC, COL1A1, COL1A2, COL3A1, COL5A1, COL5A2, COL6A3, CD47
7	TGF-beta signaling pathway: TGFB2, SP1, LTBP1, ID2, ID4, DCN, RBL1
6	Cholera – Infection: GNAS, ADCY9, ATP6V1D, ATP6V1G1, PDIA4, ARF1
6	Epithelial cell signaling in Helicobacter pylori infection: JUN, EGFR, PTPRZ1, ADAM17, ATP6V1D, ATPV1G1
6	GnRH signaling pathway: ADCY9, GNAS, MMP2, JUN, EGFR, ATF4
5	Fatty acid metabolism: ACADL, ACSL1, ACSL3, ALDH3A2, ACOX1
5	Glycan structures - biosynthesis 1: EXT1, CHST2, HS6ST2, ALG8, GALNT7
5	PPAR signaling pathway: SORBS1, ACADL, ACOX1, ACSL1, ACSL3
5	Urea cycle and metabolism of amino groups: AOC3, MAOA, ODC1, AMD1, ALDH3A2
5	Calcium signaling pathway: SLC8A1, TRPC1, ADCY9, GNAS, EGFR
5	Cytokine-cytokine receptor interaction: TGFB2, EGF, EGFR, CXCR4, CX3CL1

5	Gap junction: TUBA1A, EGF, EGFR, GNAS, ADCY9
4	Colorectal cancer: FZD7, TGFB2, JUN, EGFR
4	Wnt signaling pathway: FZD7, CCND2, WIF1, JUN

Table 4: KEGG molecular pathway-based differences detected between normal stroma from older subjects with prostate cancer and normal stroma from prostates from younger subjects without cancer (red: upgregulated, green:downregulated).

# of Features	KEGG Pathway
5	MAPK signaling pathway: TGFBR1, TBFB2, DUSP10, FAS, FLNA
5	Cytokine-cytokine receptor interaction: TGFBR1, TBFB2, CXCR4, LTB, FAS
5	Cell adhesion molecules (CAMs): VCAM, PTPRC, HLA-DPA1, HLA-C, MCAM
4	Chronic myeloid leukemia: TGBFR1, TGFB2, CBLB, SHC1
4	Insulin signaling pathway: SOCS3, CBLB, ACACA, SHC1
4	Wnt signaling pathway: SFRP2, CACYBP, DKK1, PRICKLE1
3	TGF-beta signaling pathway: TGFBR1, TGFB2, ID2
3	Focal adhesion: ACTN1, SHC1, FLNA
3	Glycine, serine and threonine metabolism: RDH11, MAOA, ALAS1
3	Tryptophan metabolism: DZIP3, AOX1, MAOA
3	Arachidonic acid metabolism: PTGS2, AKR1C3, GPX3

Results in Tables 1-4 raise several tantalizing general and specific hypotheses regarding aging-associated changes potentially relevant to prostate carcinogenesis. Specifically interesting are the marked increases in focal adhesion and other adhesion pathway members, MAPK pathway members and extracellular matrix receptor interaction pathway members in older epithelium vs younger epithelium. These observations are in some ways opposite of what might be expected and are therefore very interesting. Perhaps these changes are part of an evolved mechanism to suppress cancer globally in prostate as aging occurs, a general phenomenon that to our knowledge has not previously been considered.

We were not expecting to find such high level differences in expression between younger and older normal prostate epithelium and stroma. Because these findings have high potential importance if true, prior to attempting validation of specific pathway members through real time PCR and related studies, we have elected to expand the number of dissections and Affymetrix hybridizations, and add additional controls to the study to rule out tissue collection method artifact as the source of the above differences. Tissue samples studied in each group reported above are similar in that they are from well preserved frozen material, but they differ in that the normal prostates derive from transplant donors and were removed just after organs were removed for transplantation, and the noncancerous normal prostate tissue from older men with localized prostate cancer derived from surgery. To rule out the possibility of collection method artifact, and to better understand the role of the presence of

cancer in the older normal prostates studied, we are now expanding the number of normal prostates from transplant donors in the study, to allow statistically significant age (and race) related comparisons to be performed from samples derived from transplant donors alone. Additional samples of “normal” prostate adjacent to localized prostate cancer will also be dissected and added to the study. These studies will be completed in the coming year using funds outside of this Hypothesis Development award. As the expanded study is completed and submitted for publication, affymetrix data will be submitted to the GEO database.

Key research accomplishments

- ✓ Identification of appropriate normal tissues for study
- ✓ Determination of frozen tissue as best foundation for study
- ✓ Completion of tedious laser capture microdissections
- ✓ Completion of Affymetrix Gene Chip Hybridizations
- ✓ Confirmation of quality of RNA available from normal prostate samples collected by PI, supporting further analysis using expensive technology
- ✓ Completion of Hypothesis-generating initial analyses of epithelium and stromal comparison between younger normal prostates and noncancerous prostates from men with prostate cancer. Older normal prostate appears to take on an unexpected “protective” posture in terms of gene expression, a potentially important observation requiring urgent validation.
- ✓ Plan for additional dissections, hybridizations, and analysis to verify and publish findings underway

Reportable outcomes:

- ✓ None so far. If initial data holds true with broadening and deepening of study as described, the findings could be very helpful in advancing understanding of aging related physiology which likely to be important in increasing prostate cancer risk with aging.

Conclusions:

- ✓ Feasibility and value of studying well-curated, high quality normal prostate tissue from individual of varying age and race is confirmed.
- ✓ Specific conclusions suggested by initial analysis above to be firmed up with extension of study as described.

References: None so far

Appendices : None